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A Protein Synthesis–Dependent Late Phase of Cerebellar Long-Term Depression

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Summary

Synthesis of new protein has been shown to be required for establishment of a late phase in hippocampal long-term potentiation. Whether a similar requirement is needed to produce the late phase of long-term depression (LTD) remains to be determined. Application of transcription inhibitors or of the translation inhibitor anisomycin, immediately, but not 30 min after glutamate/depolarization conjunction, attenuated a late phase of cerebellar LTD in culture. LTD, produced in a perforated outside-out macropatch of Purkinje neuron dendrite, which lacks nuclear material, returned to baseline values with a time course paralleling that observed with protein synthesis inhibitors in intact cultured Purkinje neurons. These findings suggest that there is a distinct late phase of cerebellar LTD that is dependent upon postsynaptic protein synthesis.

Introduction

Memory storage in neural circuits appears to involve the consolidation of labile short-term memory into a more permanent long-term form (Squire, 1987). Using learning tasks in both vertebrate (Davis and Squire, 1984; Matthies, 1989) and invertebrate (Bailey et al., 1994) model systems, it has been shown that this consolidation is blocked by treatments that interfere with protein synthesis either pharmacologically or by targeted mutations within genes encoding transcription factors such as cAMP response element–binding protein (CREB) (Frank and Greenberg, 1994). It has been suggested that memories are stored in neural circuits, at least in part, through use-dependent changes in the strength of synaptic transmission (Ramón y Cajal, 1911; Hebb, 1949). Use-dependent increases in synaptic strength, called long-term potentiation (LTP; for review see Nicoll and Malenka, 1995), have also been shown to have a late phase that requires new protein synthesis (Krug et al., 1984; Stanton and Sarvey, 1984; Deadwyler et al., 1987; Abraham and Otani, 1991; Huang et al., 1994; Nguyen et al., 1994; Frey et al., 1996). Furthermore, LTP induction is correlated with the expression of a set of immediate-early genes, several of which are transcription factors (Cole et al., 1989; Dragunow et al., 1989; Wisden et al., 1990; Abraham et al., 1993; Worley et al., 1993; Yamagata et al., 1994). This suggests that there are a set of genes, the expression of which is necessary for the establishment of a late phase of LTP, a possible cellular analog of memory consolidation.

In addition to LTP, there are patterns of synaptic stimulation that have been shown to produce the converse phenomenon, use-dependent synaptic weakening, or long-term depression (LTD; for review see Linden and Connor, 1995). One particular form of LTD is found in the cerebellum. In cerebellar LTD, a persistent, input-specific attenuation of the parallel fiber–Purkinje neuron (PN) synapse is produced when parallel fiber and climbing fiber inputs to a PN are stimulated together at low frequency (Ito et al., 1982). Cerebellar LTD has been suggested to underlie certain forms of motor learning (Thompson, 1986; Ito, 1989; but see De Schutter, 1995) and is generally understood to have three initial requirements for induction (Linden and Connor, 1995). The climbing fiber contributes to LTD induction via calcium influx through voltage-gated channels (Sakurai, 1990; Konnerth et al., 1992), which is triggered by an AMPA receptor–mediated excitatory postsynaptic potential (EPSP). The parallel fibers release glutamate that acts upon both metabotropic receptors (Linden et al., 1991), specifically mGluR1 (Aiba et al., 1994; Conquet et al., 1994; Shigemoto et al., 1994), and AMPA receptors (Linden et al., 1993; Hemart et al., 1995). These initial signals are followed by stimulation of protein kinase C (PKC), the activation of which is also required (Crepel and Jaillard, 1990; Linden and Connor, 1991). The involvement of a nitric oxide/cGMP pathway in cerebellar LTD induction remains controversial, with some groups finding evidence for (Crepel and Jaillard, 1990; Shibuki and Okada, 1991; Hartell, 1994; Lev-Ram et al., 1995) and others against (Glaum et al., 1992; Linden and Connor, 1992; Linden et al., 1995) this mechanism. Cerebellar LTD is expressed, at least in part, postsynaptically as it may be detected as a decrease in response to exogenous pulses of glutamate (Ito et al., 1982) or AMPA (Linden et al., 1991).

Using cultured mouse PNs, a simplified postsynaptic preparation has been developed for the study of cerebellar LTD in which iontophoretic glutamate pulses and PN depolarization are substituted for parallel fiber and climbing fiber stimulation, respectively (Linden et al., 1991; Linden, 1994). LTD induced in this manner may be seen as a reduction of the glutamate-gated current as measured with a patch electrode attached to the PN soma. The present report has used this preparation together with three different manipulations that block protein synthesis (translation-inhibiting drugs, transcription-inhibiting drugs, and removal of somatic/nuclear material) to test the hypothesis that postsynaptic protein synthesis is necessary for a late phase of cerebellar LTD.

Results

Perforated-patch voltage-clamp recordings were made from cultured mouse embryonic PNs. Following acquisition of baseline responses to glutamate test pulses applied to two nonoverlapping sites in the PN dendritic arbor, glutamate/depolarization conjunctive stimulation was applied to site 1 but not site 2 (Figures 1A and 1B). This produced an input-specific depression of the site 1 response that persisted for the duration of the recording

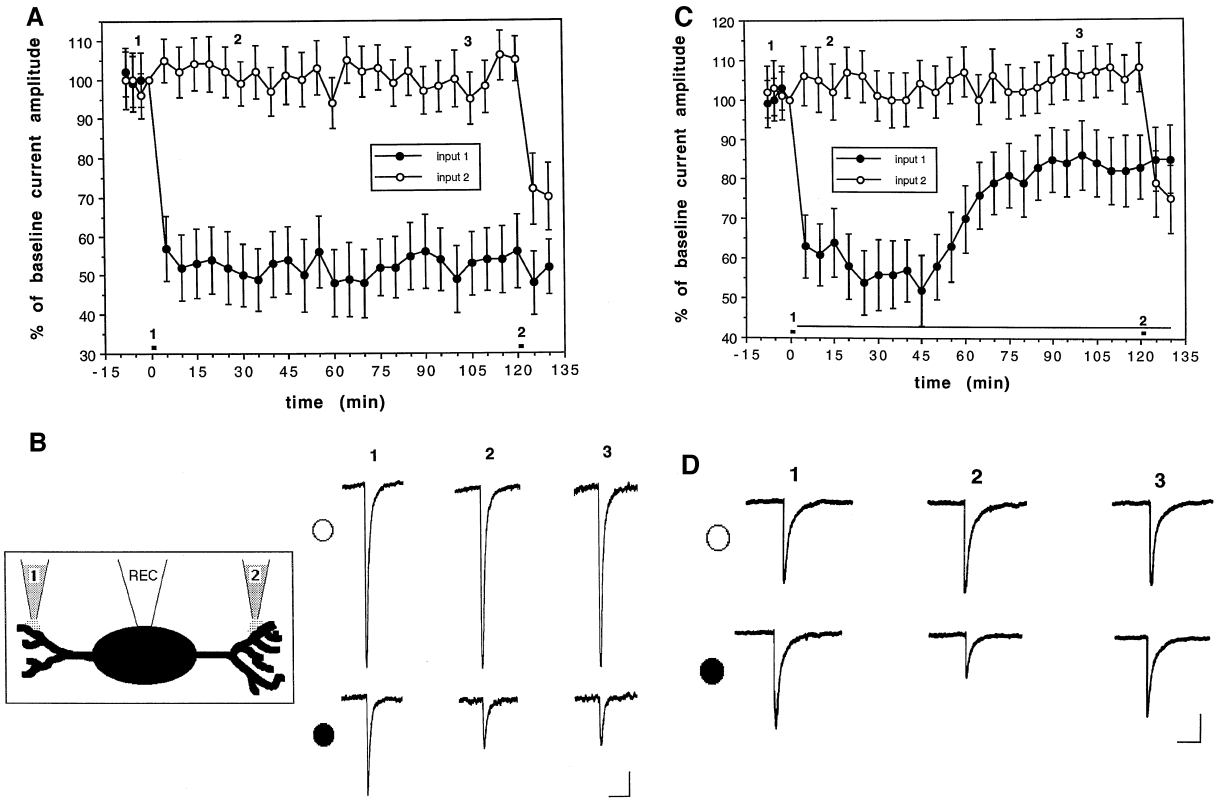

Figure 1. An Input-Specific Late Phase of Cerebellar LTD Is Attenuated by a Translation Inhibitor

(A) Six glutamate/depolarization conjunctive stimuli were applied to site 1 at t = 0 min (indicated by a heavy horizontal bar) and to site 2 at t = 120 min (also indicated by a heavy horizontal bar). Each point represents the mean ± SEM of five different PNs normalized to baseline at t = 0 min.

(B) A diagram of the recording configuration showing glutamate application to two nonoverlapping sites on the PN dendritic arbor. REC, perforated patch clamp recording electrode. Current traces from single representative PNs correspond to the times indicated in (A). Scale bars represent 2 s, 50 pA.

(C) Input-specific LTD was induced at site 1 at t = 0 min and at site 2 at t = 0 min as in (A). Anisomycin (10 μM) was applied starting at t = 2 min (indicated by the light horizontal bar). N = 5.

(D) Current traces correspond to the times indicated in (C). Scale bars represent 2 s, 50 pA.

(52% ± 8.5% of baseline at t = 10 min, 52% ± 7.0% at t = 130 min, mean ± SEM, n = 5). Conjunctive stimulation applied to site 2, 120 min after site 1, produced an input-specific depression at this location as well, although this was somewhat smaller in initial amplitude (70% ± 8.5% at t = 130 min). This result demonstrated that input-specific LTD could persist for 130 min, a necessary control to evaluate the experiments that follow.

If a late phase of cerebellar LTD requires new protein synthesis, then chemical inhibitors of transcription and translation should produce an attenuation of LTD that becomes evident with a delay following induction. Before applying these drugs, it became necessary to screen them to determine whether they had side effects that might be expected to impact cerebellar LTD independent of a direct effect on transcription or translation. As voltage-gated calcium channels (Sakurai, 1990; Linden et al., 1991; Konnerth et al., 1992) and mGluR1 receptors (Aiba et al., 1994; Conquet et al., 1994; Shigemoto et al., 1994) are known to be necessary for LTD induction, protein synthesis inhibitors were screened to determine whether they impacted either of these two signaling systems (Table 1). Fura-2 microfluorimetry was used to assess depolarization-evoked calcium influx, as an index of voltage-gated calcium channel function, and quisqualate-evoked calcium mobilization, as an index of mGluR function. The translation inhibitor anisomycin (10–30 μM) and the transcription inhibitors actinomycin D (30 μM) and 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB, 30 μM) had no significant effects upon either measure. Cyclohexamide (30 μM) and emetine (50 μM) were excluded based upon attenuation of depolarization-evoked calcium influx, and puromycin (50 μM) was excluded based upon attenuation of both measures. Doses of these drugs were chosen based upon previous work that evaluated the level of protein synthesis inhibition by measuring incorporation of radiolabeled amino acids into brain slices (Stanton and Sarvey, 1984; Nguyen et al., 1994; Osten et al., 1996).

Immediately following induction of input-specific depression at one site, anisomycin (10 μM) was bath applied, and the time course was monitored at both this site and a control site that did not receive conjunctive stimulation (Figures 1C and 1D). The depressed re-

Table 1. Effects of Protein Synthesis Inhibitors on Calcium Influx and Mobilization in Cerebellar Purkinje Neurons in Culture

Treatment	Resting (2 mM Calcium)	Depolarization Evoked	Resting (0 mM Calcium)	Quisqualate Evoked
Control	117 ± 18	450 ± 31	29 ± 9	279 ± 22
Anisomycin (10 μM)	93 ± 11	472 ± 25	33 ± 6	274 ± 25
Anisomycin (30 μM)	105 ± 22	448 ± 28	39 ± 10	283 ± 22
Actinomycin D (30 μM)	115 ± 21	455 ± 32	25 ± 8	288 ± 19
DRB (30 μM)	90 ± 15	448 ± 37	29 ± 8	265 ± 27
Cyclohexamide (30 μM)	85 ± 22	265 ± 29	32 ± 9	266 ± 24
Emetine (50 μM)	103 ± 20	244 ± 25	26 ± 8	288 ± 27
Puromycin (50 μM)	102 ± 9	199 ± 20	36 ± 10	210 ± 18

Nanomolar values are peak proximal dendritic calcium concentrations (mean ± SEM). N = 15 cells/group of which 5/group were incubated in normal (2 mM calcium containing) external saline and stimulated with a 3 s depolarizing pulse from −70 to 0 mV, and 10/group were inclubated in 0 mM calcium, 0.2 EGTA external saline and stimulated with a pressure pulse of 100 μM quisqualate (dissolved in 0 mM calcium, 0.2 EGTA saline, 6 ψ, 2 s). Cells were incubated in protein synthesis inhibitors starting 15 min prior to stimulation. Resting values were measured immediately before stimulation. Depolarization-evoked values were measured as the peak during a 30 s measuring period following the onset of depolarization, and quisqualate-evoked values were measured as the peak during a 120 s measuring period following the onset of the pressure pulse.

sponse was stable for ~50 min (61% ± 7.8% at t = 10 min, 58% ± 8.2% at t = 50 min, n = 5), but then started to increase, reaching a level of 83% ± 7.9% of baseline at t = 120 min. Anisomycin had no effect on either glutamate currents measured during the period t = 0–120 min or LTD induction at the control site at t = 120 min (compare Figures 1A and 1C, site 2). This suggests that the effect of anisomycin upon the time course of LTD at site 1 is an interaction with LTD expression and not merely a potentiation of glutamate responsivity superimposed upon the LTD time course.

Both behavioral (Davis and Squire, 1984; Matthies, 1989) and electrophysiological (Krug et al., 1984; Stanton and Sarvey, 1984; Deadwyler et al., 1987; Abraham and Otani, 1991; Huang et al., 1994; Nguyen et al., 1994; Frey et al, 1996) experiments have indicated that there is a critical temporal window in which consolidation of information storage is vulnerable to interference by protein synthesis inhibitors. To determine if this held true for cerebellar LTD, anisomycin (30 μM) was bath applied following glutamate/depolarization conjunction at a single site (Figure 2). Application of anisomycin immediately after conjunctive stimulation resulted in an attenuated late phase of LTD (85% ± 8.1% at t = 120 min, n = 6) similar to that seen in Figure 1C. However, when anisomycin was applied at t = 30 min, the time course of LTD was indistinguishable from a control (anisomycin applied at t = 30 min, 52% ± 9.0%; control, 56% ± 7.5% at t = 120 min, n = 6/group), suggesting that the critical time for late phase consolidation lies between 2 and 30 min after the beginning of conjunctive stimulation. Note that the degree of attenuation of the late phase was similar when 10 μM (Figure 1C) or 30 μM (Figure 2A) anisomycin was used, suggesting that the incomplete attenuation of the late phase by anisomycin is not due to a submaximal dose.

The drugs actinomycin D (20–50 μM) and DRB (50 μM), which inhibit transcription via different mechanisms (Laub et al., 1980), were bath applied immediately after glutamate/depolarization conjunction (Figure 3). Both drugs produced an attenuation of the late phase. The attenuation produced by these transcription inhibitors developed somewhat more slowly than that produced by anisomycin, but the degree of stable attenuation achieved was slightly greater (20 μM actinomycin D, 91% ± 7.4%; 50 μM actinomycin D, 94% ± 7.6%; 50 μM DRB, 89% ± 8.5%, at t = 120 min, n = 5/group). Actinomycin D applied at 20 and 50 μM produced effects that were indistinguishable, suggesting that 20 μM is a maximal or supramaximal dose.

The results to this point indicate that both transcription and translation inhibitors attenuate the late phase of cerebellar LTD through an action independent of mGluR1 or voltage-gated calcium channels. While these drugs are likely to be exerting their effects on LTD expression through an action on transcription and translation, there are other possibilities that remain. For example, PKC activation is necessary for LTD induction

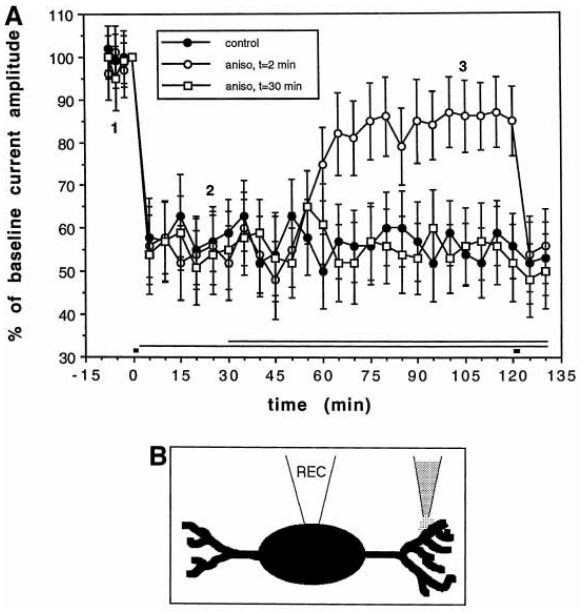

Figure 2. A Critical Period for the Consolidation of the Late Phase of Cerebellar LTD

(A) Glutamate/depolarization conjunctive stimulation was applied at t = 0 min. Anisomycin (30 μM) was applied either immediately after conjunctive stimulation (indicated by light horizontal bar at t = 2 min) or 30 min after conjunctive stimulation (also indicated by light horizontal bar).

(B) A diagram of the recording configuration used in (A), showing glutamate application to a single site on the PN dendritic arbor.

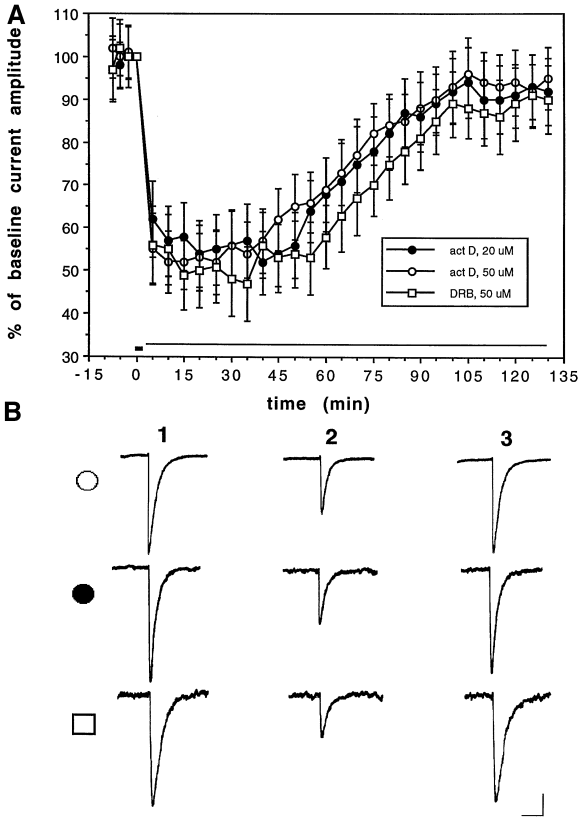

Figure 3. A Late Phase of Cerebellar LTD Is Blocked by Transcription Inhibitors

(A) LTD was induced at a single site by six glutamate/depolarization conjunctive stimuli (heavy horizontal bar at t = 0 min). Actinomycin D or DRB were bath applied immediately following conjunctive stimulation (light horizontal bar at t = 2 min). N = 5/group.

(B) Current traces correspond to the times indicated in (A). Scale bars indicate 2 s, 50 pA.

(Crepel and Jaillard, 1990; Linden and Connor, 1991), and some have claimed that a nitric oxide/cGMP/cGMP-dependent protein kinase cascade is as well (Crepel and Jaillard, 1990; Shibuki and Okada, 1991; Hartell, 1994; Lev-Ram et al., 1995; but see Glaum et al., 1992; Linden and Connor, 1992; Linden et al., 1995). It remains undetermined whether the protein synthesis inhibitors used here have an effect on these signaling pathways in cerebellar PNs. To provide an independent test of the hypothesis that a late phase of cerebellar LTD requires protein synthesis, an ultrareduced preparation in which somatic/nuclear material has been physically removed was used (Narasimhan and Linden, 1996). Perforated macropatches of membrane extracted from the primary dendrite of cultured PNs had a round or oval globular shape (~2 μm diameter) and partially protruded from the tip of the patch electrode. Application of a glutamate test pulse to a dendritic macropatch resulted in a small macroscopic inward current (range = 12–47 pA) that sometimes had resolvable single channel openings superimposed upon it (Figure 4). Glutamate/depolarization conjunction induced LTD in this preparation that was initially very similar to that seen in intact cultured PNs. However, LTD in this preparation did not establish a late phase and returned to baseline with a time course similar to that produced by transcription inhibitors in the intact PN (38% ± 8.0% at t = 10 min, 96% ± 9.0% at t = 120 min, n = 6). The transcription inhibitor actinomycin D (50 μM), applied immediately after conjunctive stimulation, had no effect upon either the initial amplitude or the attenuated time course of LTD in the dendritic macropatch (45% ± 8.6% at t = 10 min, 100% ± 7.9% at t = 120 min, n = 6). Interestingly, the translation inhibitor anisomycin (30 μM) produced a somewhat earlier attenuation of the late phase than that seen in control macropatches (control, 47% ± 8.6%; anisomycin, 84% ± 8.6% at t = 70 min; n = 6/group).

Discussion

The main finding of this report is that three different manipulations that interfere with protein synthesis, a translation-inhibiting drug, transcription-inhibiting drugs, and physical isolation of the synaptic zone from the nucleus all produced an attenuation of the late phase of cerebellar LTD in culture. Since these preparations do not depend upon stimulation of presynaptic elements for induction or monitoring, these results suggest that protein synthesis in the postsynaptic compartment contributes to expression of a late phase of cerebellar LTD.

The effects of transcription- and translation-inhibiting drugs on the late phase of LTD are likely to be specific for the following reasons: application of these drugs after conjunctive stimulation minimizes the risk that they interfere with LTD induction processes; two specific signaling mechanisms known to be required for LTD induction, mGluR1 activation and voltage-gated calcium channel function, were unaltered by these drugs; application of anisomycin had no effect on a control input; prolonged (118 min) exposure to anisomycin had no effect on the ability of a control input to show the initial stages of LTD; the effect of anisomycin was restricted to a specific time period after conjunctive stimulation. It is not clear why the attenuation of the late phase produced by anisomycin was less complete than that produced by transcription inhibitors. One possibility is that anisomycin penetrated the cell membrane more slowly, thus achieving a smaller concentration during the consolidation phase.

The lack of a late phase in the dendritic macropatch preparation provides another line of evidence for a requirement of nucleus/dendrite signaling for this process. A similar approach was taken by Frey et al. (1989) who showed that a late phase of LTP was not established in hippocampal area CA1 dendrites when they had been isolated from the cell body layer by a knife cut. The finding that anisomycin but not actinomycin D produced a somewhat earlier attenuation of the late phase in the dendritic macropatch preparation suggests, but certainly does not prove, that there are polyribosomes and mRNA present in this preparation that can contribute briefly to the expression of LTD. Polyribosomes have been shown to be present in dendrites of a number of neurons (Steward and Levy, 1982), including cerebellar PNs (Steward, 1983), where they are frequently clustered in the dendritic shaft underlying spines (Steward and

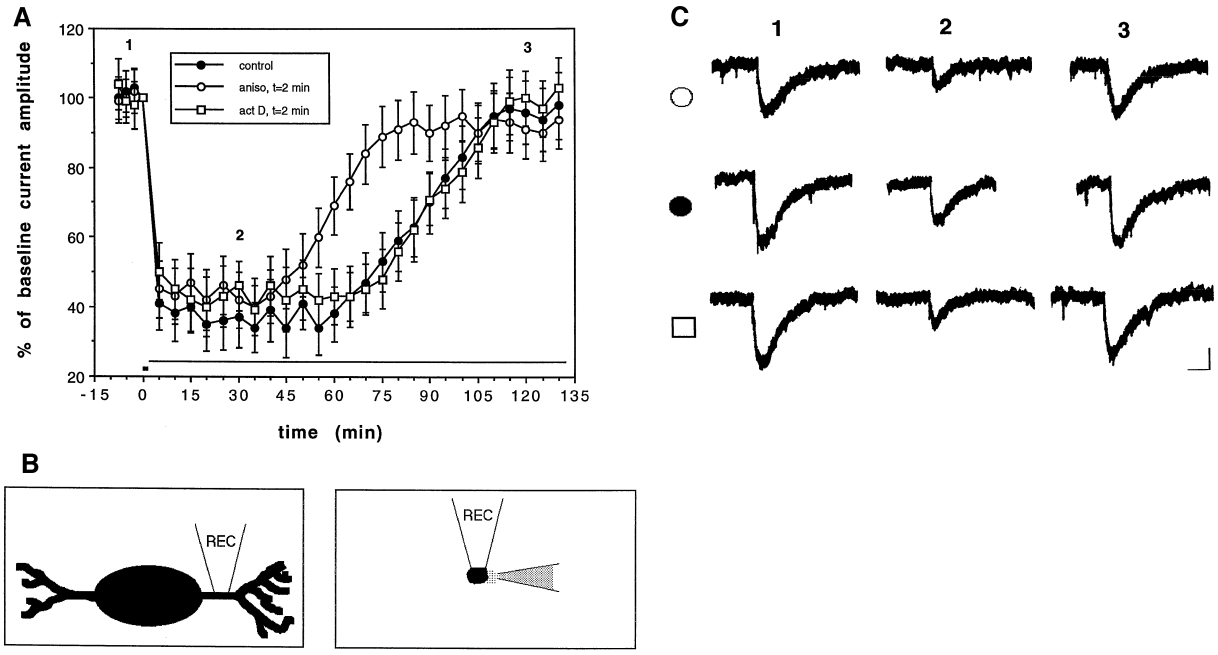

Figure 4. A Late Phase of Cerebellar LTD Is Not Established in a Dendritic Macropatch, a Preparation That Lacks Somatic/Nuclear Material

(A) LTD was induced by six glutamate/depolarization conjunctive stimuli (heavy horizontal bar at t = 0 min). Actinomycin D (30 μM) or anisomycin (30 μM) were bath applied immediately following conjunctive stimulation (light horizontal bar at t = 2 min).

(B) A diagram of the perforated dendritic macropatch preparation. Following removal of an outside-out macropatch, macroscopic currents are evoked by iontophoretic application of glutamate pulses.

(C) Current traces correspond to the times indicated in (B). Scale bars indicate 1 s, 10 pA.

Reeves, 1988). Analysis of proteins and mRNAs in dendritic macropatches should prove useful in this regard.

It has been suggested that a protein synthesis–dependent late phase of hippocampal LTP (Krug et al., 1984; Stanton and Sarvey, 1984; Deadwyler et al., 1987; Abraham and Otani, 1991; Huang et al., 1994; Nguyen et al., 1994; Frey et al, 1996) is triggered by a cAMP/cAMP-dependent protein kinase A (PKA) cascade. A late phase of LTP may be blocked by PKA inhibitors (Matthies and Reymann, 1993; Huang and Kandel, 1994) and mimicked by exogenous analogs of cAMP (Frey et al., 1993; Slack and Walsh, 1995) or an adenylate cyclase activator (Huang et al., 1994). A similar suggestion has been made (Schacher et al., 1988, 1993; Ghirardi et al., 1992) for a protein synthesis–dependent late phase of long-term facilitation of a sensorimotor synapse in the marine mollusk Aplysia (Montarolo et al., 1986; Bailey et al., 1994). One potential mechanism by which PKA activation could be transduced into changes in gene expression is by phosphorylation of the transcription factor CREB (Frank and Greenberg, 1994). In support of this notion, it has been reported that mice which lack the α and δ isoforms of CREB are impaired in both a late phase of LTP and in long-term (but not short-term) memory of certain behavioral tasks (Bourtchuladze et al., 1994). Similarly, induction of a dominant negative CREB transgene was reported to block long-term, but not short-term memory of an olfactory learning task in Drosophila (Yin et al., 1994). In the Aplysia sensorimotor synapse, injection into the sensory cell nucleus of an excess of CRE oligonucleotide (Dash et al., 1990) can block long-term, but not short-term facilitation. It should be noted that CREB may be phosphorylated by kinases other than PKA, including calcium/calmodulin-dependent protein kinases (Frank and Greenberg, 1994; Deisseroth et al., 1996). In the present system, we have no evidence either for or against PKA or calcium/calmodulin-dependent protein kinase involvement in the late phase of cerebellar LTD. Loading of PNs with peptide inhibitors of PKA or calcium/calmodulin-dependent protein kinase had no effect on cerebellar LTD induction or expression monitored for 40 min after glutamate/depolarization conjunctive stimulation (D. J. L., unpublished data).

While this report demonstrates a protein synthesis–dependent late phase of LTD in the mammalian brain, similar phenomena have been reported using invertebrate model systems. Long-term heterosynaptic inhibition of the Aplysia sensorimotor synapse may be produced by repeated applications of the molluscan hormone FMRFamide (Montarolo et al., 1988). Both the late phase of this synaptic inhibition (as measured by the EPSP amplitude 24 hr later) and the reduction in presynaptic varicosities that are presumed to underlie this effect are blocked by protein synthesis inhibitors (Bailey et al., 1992) or PKA inhibitors (Wu and Schacher, 1994). Long-term adaptation of the crayfish neuromuscular junction is a phenomenon in which prolonged stimulation of presynaptic fibers at 5–7 Hz produces transformation of this synapse from a phasic to a tonic type, one aspect of which is a reduction in EPSP amplitude (for review see Linden and Connor, 1995). Application

of the translation inhibitor cyclohexamide during this stimulation attenuated long-term adaptation and had no significant effect on unstimulated control synapses (Nguyen and Atwood, 1990).

Previous work from this laboratory has identified an earlier temporal switching point in the consolidation of cerebellar LTD. Inhibition of phospholipase A_2 converted LTD to a form of short-term depression that returns to baseline within ~30 min (Linden, 1995). This effect was reversible by coapplication of the natural product of phospholipase A_2 action, free unsaturated fatty acids, and was independent of any actions on mGluR1 or voltage-gated calcium channels. Free unsaturated fatty acids exert a portion of their effect by stimulation of PKC, the activation of which is necessary for LTD induction. At present, it is unclear whether recruitment of phospholipase A_2 or PKC signaling is necessary to trigger the protein synthesis–dependent late phase seen in the present report. In other model systems, it has been suggested that a late phase of use-dependent synaptic modification may be induced independently of the early phase (Emptage and Carew, 1993; Frey et al., 1995).

The existence of a protein synthesis–dependent late phase of cerebellar LTD brings about the question of which genes are expressed to mediate this phenomenon. While no specific information is available on this point, two important constraints should be noted. First, as cerebellar LTD in this (and other) preparations appears to be postsynaptically expressed, the relevant transcripts or proteins must be capable of influencing dendritic function, specifically, some form of down-regulation in the response of dendritic AMPA receptors. Second, the late phase of LTD in this preparation remains input specific. While this might mean that the relevant mRNA or protein(s) are targeted to specific dendritic regions (Steward and Banker, 1992), it should be emphasized that these signals could also be widely distributed but rely upon conjunction with a separate, spatially constrained signal to confer input specificity (Linden, 1994; Sossin, 1996). High resolution in situ hybridization and immunohistochemistry performed in conjunction with input-specific cerebellar LTD induction in culture holds promise for addressing this question.

Experimental Procedures

Mouse cerebellar cultures were prepared and maintained according to the method of Schilling et al. (1991). Cultures were maintained in vitro for 10–16 days at the time of use in electrophysiological experiments. Patch electrodes attached to PN somata were filled with a solution containing Cs_2SO_4 (95 mM), CsCl (15 mM), $MgCl_2$ (8 mM), HEPES (10 mM), adjusted to pH 7.35 with CsOH. Electrode tips were filled with a small amount of this solution, and the shanks backfilled with this solution were supplemented with amphotericin B (Sigma) at a concentration of 300 μg/ml (Rae et al., 1991). Stable access resistance of <10 MΩ could be obtained within 10 min of gigaseal formation. A holding potential of −70 mV was imposed. Iontophoresis electrodes (1 μm tip diameter) were filled with 10 mM glutamate (in 10 mM HEPES, adjusted to pH 7.1 with NaOH) and were positioned ~20 μm away from large caliber dendrites. Test pulses of glutamate were delivered using negative current pulses (600–800 nA, 30–110 ms duration) applied at a frequency of 0.05 Hz. After acquisition of baseline responses, six conjunctive stimuli were applied, each consisting of a glutamate test pulse combined with a 3 s long depolarization step to 0 mV. Cells were bathed in a solution that contained NaCl (140 mM), KCl (5 mM), $CaCl_2$ (2 mM), $MgCl_2$ (0.8 mM), HEPES (10 mM), glucose (10 mM), tetrodotoxin (0.0005 mM), and picrotoxin (0.01 mM), adjusted to pH 7.35 with NaOH, which flowed at a rate of 0.5 ml/min. Patch electrodes were pulled from N51A glass and polished to yield a resistance of 3–5 MΩ when measured with the internal and external salines described above. Membrane currents evoked by application of glutamate were recorded with an Axopatch 200A amplifier in resistive voltage-clamp mode.

The technique used for perforated dendritic macropatch recording was a variant of that described by Levitan and Kramer (1990). Patch electrodes were fabricated as described above, with the exception that they were somewhat smaller, yielding a resistance of 5–6 MΩ. Following gigaseal formation, the recording electrode was axially retracted very slowly (~2 μm/min) to form the perforated outside-out macropatch (Narasimhan and Linden, 1996). This procedure was successful ~30% of the time. Glutamate pulse application, external saline composition, current recording, and the glutamate/depolarization conjunction protocol were the same as used for intact PNs. Experiments were conducted at room temperature. Protein synthesis inhibitors were dissolved in ddH_20 prior to dilution in external saline. All drugs were applied by switching the bath solution to one containing the drug at a point upstream from the bath chamber.

Ratio imaging of intracellular free calcium was accomplished by fura-2 microfluorimetry using a cooled CCD camera system as previously described (Linden, 1995). The quisqualate-evoked calcium mobilization measure may reflect a contribution of both mGluR1 and mGluR5 isoforms, both of which are linked to production of inositol-1,4,5-trisphosphate and the consequent liberation of calcium from intracellular stores. In addition, both influx and mobilization measures will contain within them a portion of the signal deriving from calcium-induced calcium release (Llano et al., 1994).

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